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Development and Validation of Swine Embryonic Stem Cells: a Review

Matthew B. Wheeler

Department of Animal Sciences, Laboratory of Molecular Embryology, University of Illinois, Urbana, IL 61801, USA.

Abstract. The establishment of embryonic cell lines from swine should be useful for studies of cell differentiation, developmental gene regulation and the production of transgenics. This paper summarizes the establishment of porcine (*Sus scrofa*) embryonic stem (ES) cell lines from preimplantation blastocysts and their ability to develop into normal chimaeras. ES cells can spontaneously differentiate into cystic embryoid bodies with ectodermal, endodermal, and mesodermal cell types. Further, culture of ES cells to confluence or induction of differentiation with retinoic acid or dimethylsulfoxide results in morphological differentiation into fibroblasts, adipocytes, and epithelial, neuronal, and muscle cells. These ES cells have a normal diploid complement of 38 chromosomes. Scanning electron microscopy of the ES cells reveals a rounded or polygonal, epithelial-like cell with numerous microvilli. The differentiation of these embryonic cell lines into several cell types indicates a pluripotent cell. Furthermore, chimaeric swine have been successfully produced using such ES cells.

Introduction

The ability to isolate embryonic cells directly from preimplantation mouse embryos and to maintain them *in vitro* has provided a powerful research tool. The ability of embryonic cells to differentiate into a wide variety of cell types has lead to their designation as embryonic stem (ES) cells. ES cells have been derived from the inner cell mass of embryos at the blastocyst stage (Evans and Kaufman 1981; Martin 1981; Bradley *et al.* 1984; Doetschman *et al.* 1985). These cells are able to produce all tissues of a new individual (Nagy *et al.* 1993). Once isolated, ES cells may be grown *in vitro* for many generations. This produces an unlimited number of identical cells capable of developing into fully formed adult chimaeras (Evans and Kaufman 1981; Martin 1981; Bradley *et al.* 1984; Wobus *et al.* 1984; Robertson 1987*a*). The isolation of ES cells has developed from pioneering studies performed with embryonal carcinoma (EC) cell lines (Stevens 1970; Evans and Kaufman 1981; Martin 1981). Embryonal carcinoma cells were originally derived from embryonic cell tumours or teratocarcinomas (Stevens 1970). Rossant and Papaioannou (1984) showed that both EC and ES cells may differentiate *in vitro* into similar cell types. However, the formation of chimaeric embryos exhibiting phenotypically normal development using EC cells is usually low (Papaioannou *et al.* 1979; Rossant and McBurney 1982), whereas ES cells are more efficient at producing chimaeric mice (Bradley *et al.* 1984). This combined evidence demonstrates that ES cells are a better model than EC cells for studying early mammalian development, and differentiation and for producing transgenic individuals.

The ability of ES cells to serve as vectors for the transfer of foreign DNA in the production of transgenic mice (Gossler *et al.* 1986; Robertson *et al.* 1986) is proving to be a significant aspect of biomedical research. ES cell-mediated transgenesis has some distinct advantages over other transgenic methods. First, the efficiency of producing transgenic animals may be significantly increased with this technology (Gossler *et al.* 1986; Rexroad and Pursel 1988). Secondly, these cells can be transformed *in vitro* with foreign DNA (Thomas and Capecchi 1987; Capecchi 1989; Robertson 1991). Genetically transformed ES cells can be selected and individual cell lines (clonal lines) can be derived from a single cell. Once isolated, these individual cell lines can be screened for recombination (homologous or non-homologous) of exogenous DNA into chromosomal DNA. This provides the opportunity to establish the stable incorporation of the desired gene before chimaera production (Gossler *et al.* 1986; Thomas and Capecchi 1987). When DNA-transformed ES cells are incorporated into the gonads and participate in the production of sperm and eggs, the offspring that are produced by such chimaeric individuals will be transgenic (Bradley *et al.* 1984; Robertson 1987*a*, 1991).

Production of transgenic pigs from DNA-transformed, individually-derived and screened ES cell lines would allow large numbers of genetically altered pigs to be established. These pigs, if derived from a single ES cell line, would have the transgene integrated into a specific location within the genome. In addition, if homologous recombination is utilized, a single copy of the transgene could be inserted into the genome at a specific site. The use of homologous recombination and site-directed

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mutagenesis can provide precise changes in endogenous genes (Thomas and Capecchi 1987). Insertion of single copies of transgenes could circumvent the problems encountered by integration of multiple gene copies as has been seen with many of the GH transgenic pigs (Rexroad and Pursel 1988). This could potentially allow development of stable, genetically altered strains or lines of swine. Additionally, it may be possible to screen for tissue specific expression of the inserted gene before insertion of the embryonal cells into the embryo (Smithies *et al.* 1985). It also may be possible, with selection markers, to increase the frequency of homologous recombination. This would allow replacement of existing genes with genetically-altered genes designed to improve livestock rather than insertion of genes randomly into the host genome.

Establishment of ES cells from a wide variety of species will allow more flexibility in gene regulation studies and developmental biology research. In addition to the mouse, isolation of embryo-derived cell lines has also been reported from preimplantation embryos of the Syrian golden hamster (Doetschman *et al.* 1988; Piedrahita *et al.* 1990c), rabbit (Giles *et al.* 1991, 1993; Neimann and Strelchenko 1994), mink (Sukoyan *et al.* 1992, 1993), pig (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Pichrahita *et al.* 1990*a*, 1990*b*; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Onishi and Youngs 1993*a*, 1993*b*; Talbot *et al.* 1993*a*, 1993*b*; Gerfen and Wheeler 1994), cattle (Strelchenko *et al.* 1991; Stringfellow *et al.* 1991; Saito *et al.* 1992; Simms and First 1993; Cherny and Merei 1994; Stice *et al.* 1994; Strelchenko and Stice 1994), and sheep (Handyside *et al.* 1987; Flechon *et al.* 1990; Piedrahita *et al.* 1990*a*; Tsuchiya *et al.* 1994). Swine offer some distinct advantages over other species because they are immunologically and physiologically more similar to humans and thus serve as a better research model (Phillips and Tumbleson 1986). One particularly important application of this technology is the use of swine as biological reactors or factories to produce human proteins necessary for treatment of genetic or other diseases. Transgenic swine have been shown to produce more than 1 g L^{-1} of a foreign heterologous milk protein (Wall *et al.* 1991). Since swine may potentially produce up to 10 kg of milk per day, and lactation lasts 7 weeks, one sow may produce 1 kg of a human protein such as clotting Factor IX during her lactation (Wall *et al.* 1991). Another important application of transgenic swine is to produce cells, tissues or organs which contain human antigens or proteins for xenografting.

Finally, by utilizing genetically altered ES cells in nuclear transfer strategies, it may be possible to establish large numbers of genetically identical pigs. Identical animals would be a great advantage to researchers interested in the effects of identified genes, to pharmaceutical companies interested in the production of gene produ such as biological hormones, proteins, tissue antige and antibodies, to agricultural producers for selecti of disease resistance, uniformity in production tra and reproductive performance and to basic scienti and developmental biologists for studies regarding ge expression and growth.

Summary of Recent Studies

In this paper we review the establishment of stab embryonic stem cell lines directly from preimplantatic porcine (*Sus scrofa*) blastocysts, the differentiation these ES cells into numerous cell types, and the embryon development of swine ES cells into viable chimaer pigs (M. B. Wheeler, L. A. Rund, A. M. Davis, G. Bleck, M. L. Roach, V. L. Jarrell, B. R. White and L. Bidner, unpublished data).

Methods

Embryo collection, culture and stem cell isolation. ES cells were isolated from porcine blastocysts and maintaine in culture (Wheeler, Rund, Davis, Bleck, Roach, Jarrell White and Bidner, unpublished data). Embryos wer cultured individually on mitomycin C-inactivated mous embryonic fibroblasts (STO) monolayers with 2 mL o stem cell medium (SCM; Robertson 1987*b*; Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data).

After 7–9 days in culture, the whole embryonic cell colony was plucked off the feeder layer with a fine glass pipette and partially dis-aggregated in a buffered solution of trypsin containing ethylenediamine tetraacetic acid (trypsin–EDTA) (Robertson 1987*b*). At passages 2–4, the cells were plated onto a dish with no feeder layer with buffalo rat-liver (BRL)-conditioned stem cell medium (CSCM) (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). ES cells in CSCM have been maintained for up to 44 passages and extensively characterized.

Preparation of porcine ES cells for chimaera production. ES cell colonies were plucked with a fine pipette to dislodge them from the underlying cells. The colonies were then transferred to a solution of trypsin–EDTA. The colonies were placed in CSCM to neutralize the trypsin. The ES cells were gently pelleted by centrifugation and then re-suspended in Dulbecco's phosphate-buffered saline (D-PBS; Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data).

Production of chimaeras. Differentiation of pluripotent ES cells *in vivo* was tested by their ability to participate in the formation of chimaeric offspring. Embryos at the morula, blastocyst and expanded blastocyst stage were injected with ES cells by means of a glass injection needle attached to a micromanipulator as previously described

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(Favero and Wheeler 1991). After injection, the embryos were immediately transferred to recipient gilts.

Chimaeric embryos were produced using two coat colour markers: Meishan (black hair with black skin pigmentation) ES cells were injected into Duroc (red–brown hair with pink skin pigmentation) embryos. These combinations allowed for easy visual detection of chimaeric animals. Resultant individuals have patches of different skin and hair colour derived from each embryonic cell lineage.

Results

The production of chimaeric pigs from porcine (*Sus scrofa*) ES cells is described. Participation of blastocyst-derived cells in embryonic development as well as in differentiation of these embryonic cell lines into a variety of cell types indicates a pluripotent stem cell. The ES cell lines derived from pig blastocysts have similar morphology to mouse ES cells. Porcine ES cells grow in colonies with diameters that range from 0·08 mm to >1·5 mm. The individual cells making up these colonies are small (8–12 μm in diameter), round and dark yet translucent. Their nucleus contains several prominent nucleoli and makes up ~80% of the cell volume. Scanning electron micrographs of the ES cells reveal a rounded or polygonal cell with close association of cells to one another; irregular surfaces and microvilli are evident on the outermost cells of the colony (Gerfen and Wheeler 1994). The doubling time of stable pig ES cell lines ranges from 18 h to 48 h. ES cells can spontaneously differentiate into cystic embryoid bodies with ectodermal, endodermal, and mesodermal cell types (Gerfen and Wheeler 1994). Furthermore, culture of ES cells to confluence or induction of differentiation with 1 μM retinoic acid or 1% dimethylsulfoxide results in the morphological differentiation into fibroblasts, adipocytes, epithelium, neurons and muscle cells. Karyotype analysis of the ES cells revealed a normal diploid complement of 38 chromosomes.

Results have shown a high percentage (96%) of recipient embryos that received ES cells survived micromanipulation. Furthermore, embryo transfer efficiencies (40%) of micromanipulated embryos were similar to those reported for non-micromanipulated porcine embryos. At birth, 72% of offspring produced after micromanipulation and transfer of embryos exhibited coat-colour chimaerism. Coat colour patterns varied with the individual but included single or multiple areas of black hair, dorsal and ventral striping or a combination of these patterns. Furthermore, skin biopsy revealed chimaerism at the level of the individual hair follicles. Performance testing of coat colour chimaeric swine showed growth rates, measured by average daily gain (ADG, in g) of the chimaeras to be intermediate between the Duroc (embryo donor) and the Meishan (ES cell line donor) age-matched control pigs. The ADG of the chimaeras was significantly different ($P < 0·05$) from both the Duroc and Meishan control pigs. The chimaera growth rate was about 90% that of the Duroc pigs and higher ($P < 0·001$) than that of Meishan pigs (Ellis and Wheeler, unpublished data). Analysis by means of polymerase chain reaction (PCR) of the cholesterol-7α-hydroxylase (C7αH) genetic system (Davis and Wheeler 1994) is currently under way to establish the percentage of chimaerism in the individual tissues (Davis, Rund, Bleck and Wheeler, unpublished data). Chimaeric individuals should contain C7αH breed-specific polymorphisms from both Meishan and Duroc genotypes. Analysis of germ line chimaerism in these pigs has not been completed at this time.

Discussion

The participation of embryo-derived cells in embryonic development as well as differentiation of these embryonic cells into several distinct cell types indicates a pluripotent cell that is of ES cell origin. Numerous methods have been reported for the validation of ES cell lines from mammals. Studies in mice (Martin and Evans 1975; Evans and Kaufman 1981; Bradley *et al.* 1984; Wobus *et al.* 1984; Doetschman *et al.* 1985; Risau *et al.* 1988) and in Syrian Golden Hamsters (Doetschman *et al.* 1988) have used cell morphology, biochemical markers, the ability to differentiate into various cell types and tissue types and participation in embryonic development as criteria for validating the stem cell nature of embryonic cell lines. In swine, there have been several reports of the isolation of embryonic cell lines (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990*a*, 1990*b*; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Gerfen and Wheeler 1994) and inner cell mass cells (Onishi and Youngs 1993*a*, 1993*b*; Talbot *et al.* 1993*b*) from blastocysts. The criteria used for evaluation of undifferentiated ES cell phenotype in these studies have included morphology (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990*a*, 1990*b*; Gerfen *et al.* 1991), ability to differentiate *in vitro* into various cell types and tissue types (Evans *et al.* 1990; Notarianni *et al.* 1990; Piedrahita *et al.* 1990*a*, 1990*b*; Gerfen *et al.* 1991; Gerfen and Wheeler 1994), limited embryonic cell analysis of biochemical markers via immunocytochemistry (Evans *et al.* 1990; Piedrahita *et al.* 1990*a*) and enzyme assay (Talbot *et al.* 1993*a*; Gerfen and Wheeler 1994).

The most appropriate validation for establishing definitive ES cells is the participation of such cells in embryonic development resulting in the production of live offspring. However, none of the reported cell lines have produced

chimaeric offspring after the re-introduction of these cells into pre-implantation porcine embryos. Chen and Wu (1993) reported re-introduction of ES-like cells into blastocysts and the production of live young. However, chimaerism of the offspring was not definitively established. Furthermore, the reported ES-like cells (Evans *et al.* 1990; Notarianni *et al.* 1990, 1991; Piedrahita *et al.* 1990*a*, 1990*b*; Strojek *et al.* 1990; Gerfen *et al.* 1991; Chen and Wu 1993; Gerfen and Wheeler 1994) differ morphologically from those isolated, cultured and re-introduced into swine embryos by our group. Recently, we have reported that a high percentage (96%) of recipient embryos receiving ES cells survived micromanipulation and that 40% of these produced live offspring; at birth, 72% of these pigs exhibited observable coat-colour chimaerism (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). This is, to our knowledge, the first report of the production of validated chimaeric swine from ES cells.

We have used coat colour markers as previously reported in mice (Mintz 1965) to identify chimaeric offspring. Coat colour markers offer a visually observable system for distinguishing chimaerism (McLaren 1976). Chimaeric pigs were produced by means of two coat colour markers: Meishan (black hair with black skin pigmentation) ES cells were injected into Duroc (red–brown hair with pink skin pigmentation) embryos. These coat colour combinations allowed for easy visual detection of chimaeric animals. Resultant individuals have patches of different skin and hair colour derived from each embryonic cell lineage. Microscopic examination has indicated that chimaerism may extend to the individual hair follicles in the mouse (McLaren and Bowman 1969). Examination of skin from chimaeric swine revealed chimaerism at the level of the individual hair follicles. Coat colour patterns varied between individuals. Patterns included single or multiple areas of black hair, dorsal and/or ventral striping or a combination of these patterns (Wheeler, Rund, Davis, Bleck, Roach, Jarrell, White and Bidner, unpublished data). Similar coat colour patterns have been previously reported for chimaeric mice (Mintz 1967).

Conclusions

The establishment of ES cells from swine should be useful for studies of cell differentiation, development, gene regulation and the production of transgenic swine. ES cell-mediated gene transfer has not been used in domestic livestock mainly because of the lack of established, stable ES cell lines. There are two barriers to overcome to produce transgenic pigs with this technology: (*1*) establishment of undifferentiated ES cell lines; and (2) the successful transformation of the ES cells with the 'foreign' gene(s). In this paper we summarized progress toward overcoming the first of these barriers, that is, the isolation of undifferentiated porcine ES cell lines and production o the first chimaeric pigs from cultured ES cells.

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